Regulatory Mechanisms in the Human Leukocyte

I. The Feedback Control of Deoxycytidylate Deaminase

By Robert Silber

Deoxycytidylate deaminase, which catalyzes the conversion of deoxycytidylate to deoxyuridylate via the reaction sequence

\[ \text{dCMP}^* + H_2O \rightarrow \text{dUMP} + \text{NH}_3 \]

has been extensively investigated in the developing chick embryo\(^2,3\) and in several mammalian tissues.\(^4,5\) Variations with the degree of mitosis in regenerating liver\(^4\) and the presence of high levels in rapidly proliferating neoplastic\(^6\) and embryonic tissues have suggested a significant role for this enzyme in cell proliferation.\(^2\)

In a previous study dCMP deaminase activity has been detected in human leukocytes.\(^7\) This paper reports on the characterization of dCMP deaminase in a partially purified preparation and on its feedback control in normal and leukemic leukocytes. In the course of these investigations an active nucleoside deaminase was detected in human leukocytes. Data are presented on the properties and levels of this enzyme in normal and leukemic cells.

Materials and Methods

dCMP, dCTP, CMP, dC, cytidine, TMP, dTTP, and dUMP were obtained from Calbiochem; dUTP was purchased from Sigma Chemical Co. Leukocytes were isolated from peripheral blood of normal subjects and from patients with leukemia by a previously described procedure.\(^8\) "Normal leukocytes" were isolated from laboratory personnel. These preparations generally contained from 75 to 90 per cent granulocytes. Patients with leukemia were studied only during relapse. In order to obtain relatively homogeneous preparations of a given leukemic cell type, only patients with a leukocyte count in excess of 50,000 cells/mm\(^3\) were included in the study. The differential count of the leukocytes isolated from the patients with CLL showed over 85 per cent small lymphocytes. Only those chronic myelocytic leukemia patients with leukemia counts above 40,000 cells/mm\(^3\) and with more

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*Abbreviations

dCMP: deoxycytidine 5'-PO\(_4\)
dUMP: deoxyuridine 5'-PO\(_4\)
mdCMP: methyl deoxycytidine 5'-PO\(_4\)
dTTP: deoxythymidine 5' triphosphate
dCTP: deoxycytidine 5'-triphosphate

pHMB: parahydroxymercuribenzoate
AmBu: ammonium sulfate: butanol
AL: acute leukemia
CML: chronic myelocytic leukemia
CLL: chronic lymphocytic leukemia

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than 30 per cent immature granulocytic forms were studied. At least 75 per cent blast forms were present in the leukocytes isolated from the patients with acute leukemia. No patients receiving chemotherapy are included in this report.

The cells were suspended in 3 volumes of 0.08 M potassium phosphate buffer, pH 7.5, and homogenized in a Virtis homogenizer at 45,000 r.p.m. for 3 minutes. The homogenate was centrifuged at 10,900 g for 15 minutes and the supernatant was used for enzyme assays or purification procedures. Ammonium sulfate fractionation was carried out by the addition of solid ammonium sulfate at 4 C. Protein concentration was determined by the biuret reaction.

dCMP, cytidine or dC deaminase activities were determined in crude homogenates by the assay of Maley and Maley. The reaction was started by the addition of enzyme (0.1 to 0.7 ml.) containing 1 to 5 mg. of protein. At 0, 15, 30 and 60 minutes, 0.1 ml. aliquots were removed and after 40-fold dilutions in 5 per cent TCA, their extinction was measured in a Gilford spectrophotometer at a wavelength of 290 m. The purified enzyme was assayed at pH 7.8 by the direct spectrophotometric method of Scarano, using mdCMP as the substrate. Specific activities are expressed as μmoles/hr./mg. protein.

RESULTS

Identification of Reaction Products

When leukocyte homogenates were incubated with dCMP or dC, a decrease in extinction at 290 m occurred. This could represent the formation of dUMP or dU by either of the reaction sequences outlined below:

\[
\begin{align*}
dCMP & \xrightarrow{1} dUMP \\
dC & \xrightarrow{1} dU
\end{align*}
\]

An experiment was performed to identify the reaction products and intermediates. One-tenth ml. aliquots were removed from the reaction mixture at 0, 90 and 160 minutes of incubation and spotted on Whatman #1 paper for descending chromatography in the Ammonium sulfate:Butanol or Isopropanol:HCl:H2O systems. Both of these systems separated dCMP, dUMP, dC and dU. When dCMP was used as the substrate, the only ultraviolet “quenching” spot on the chromatogram at 0 minutes corresponded to dCMP (Rf 0.50 in the Am:Bu and 0.66 in Isoprop:HCl:H2O). At 20 minutes two quenching spots were observed on each chromatogram, the new spot corresponding to dUMP (Rf 0.35 in Am:Bu and 0.79 in Isoprop:HCl:H2O). Upon elution in 0.001 M HCl the A290 of this spot was 0.470. At the end of 60 minutes the dCMP spot was barely detectable, while the dUMP spot had increased in intensity and had an absorbance of 0.780 at 260 m. Since neither dC nor dU were detected as intermediates, it appeared that a direct conversion of dCMP to dUMP was taking place. When dC was added as substrate in the incubation mixture, only dU could be detected at the end of the reaction.

Purification and Properties of dCMP Deaminase

Prior to investigating substrate and inhibitor kinetics, the enzyme was purified from CLL cells by the procedure of Scarano and associates (Table 1). The first four steps of this procedure were adapted to leukocytes with no modifications except for scaling down the quantity of material processed in view of the limited starting material available. The almost complete loss of
Table 1.—Purification of dCMP Deaminase

<table>
<thead>
<tr>
<th>Step</th>
<th>Vol. (ml.)</th>
<th>dC</th>
<th>dCMP</th>
<th>Total Units $\times 10^{-2}$</th>
<th>dC</th>
<th>dCMP</th>
<th>Protein μmoles/mg.</th>
<th>Specific Activity μmoles/mg.</th>
<th>Yield</th>
<th>dC</th>
<th>dCMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. High speed supernatant</td>
<td>25</td>
<td>170</td>
<td>266</td>
<td>42.5</td>
<td>66.5</td>
<td>28.0</td>
<td>0.6</td>
<td>0.9</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2. NH₄SO₄ fractionation</td>
<td>10</td>
<td>199</td>
<td>406</td>
<td>19.9</td>
<td>40.6</td>
<td>25.5</td>
<td>0.8</td>
<td>1.6</td>
<td>47</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>(90-60%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Ethanol fractionation I</td>
<td>6</td>
<td>275</td>
<td>390</td>
<td>16.6</td>
<td>23.4</td>
<td>23.0</td>
<td>1.2</td>
<td>1.7</td>
<td>40</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>(30-50%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Ethanol fractionation II</td>
<td>6</td>
<td>185</td>
<td>245</td>
<td>11.0</td>
<td>14.8</td>
<td>5.8</td>
<td>3.2</td>
<td>4.2</td>
<td>26</td>
<td>21</td>
<td></td>
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</tbody>
</table>

*Enzyme purified from CLL lymphocytes. Leukocytes were isolated from 450 ml of blood with a WBC of 418,000 cells/mm³. The starting preparation contained 97 per cent lymphocytes, 2 per cent large lymphocytes and 1 per cent polymorphonuclear granulocytes. The cells were suspended in 0.05 M potassium phosphate buffer pH 7.8, containing 0.01 M mercaptoethanol. The suspension was homogenized for 3 minutes at 45,000 r.p.m. with a Virtis homogenizer. The homogenate was centrifuged for 15 minutes at 10,000 g and the supernatant fluid used for further purification studies.

Table 2.—Separation of dCMP Deaminase from Nucleoside Deaminase by Ammonium Sulfate Fractionation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific Activity*</th>
<th>Substrate</th>
<th>Ratio of dCMP/dC Deamination</th>
<th>Ratio of Cytidine/dC Deamination</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-33</td>
<td>2.8</td>
<td>3.0</td>
<td>0.93</td>
<td>0.97</td>
</tr>
<tr>
<td>33-40</td>
<td>8.6</td>
<td>8.7</td>
<td>0.99</td>
<td>1.0</td>
</tr>
<tr>
<td>40-50</td>
<td>18.0</td>
<td>8.2</td>
<td>2.2</td>
<td>0.91</td>
</tr>
<tr>
<td>50-60</td>
<td>4.2</td>
<td>0.7</td>
<td>6.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Expressed in μmoles/hr./mg. protein. Leukocytes were obtained from a patient with CLL whose WBC was 265,000 cells/mm³. The preparation contained 98 per cent lymphocytes.

activity that followed chromatography on DEAE cellulose or gel filtration in the absence of dCTP made further purification impractical. Although this purification scheme failed to separate dCMP deaminase from the nucleoside deaminase, the individual nature of two enzymes was established in three ways. The first two methods consisted of heating the homogenate to 65°C for 3 minutes or lowering the pH to 5 with 1 M acetic acid. Either of these procedures resulted in complete loss of dCMP deaminase activity but had no effect on the nucleoside deaminase. Separation of the enzymes was accomplished by ammonium sulfate fractionation. Table 2 shows the progressive enrichment of dCMP deaminase over nucleoside deaminase activity during precipitation by increasing concentrations of ammonium sulfate, suggesting that dCMP and nucleoside deaminase activities are catalyzed by two different proteins. Throughout this fractionation, cytidine and dC were deaminated at the same relative rate, suggesting catalysis by a single enzyme. This observation is analogous to the finding that nucleoside deaminase from mouse kidney has equal activity with ribonucleosides or deoxyribonucleosides.¹⁰

The effect of dTTP and dCTP on enzyme kinetics was investigated in a series of experiments with the leukocyte dCMP deaminase purified through step 4 (Table 1). As has been reported for this enzyme in other tissues, dTTP...
Fig. 1.—Effects of inhibitor (dTTP) and activator (dCTP) on substrate-velocity curve of dCMP deaminase. Each cuvette contained 0.1 molar phosphate buffer pH 7.8, enzyme (specific activity 10 μmoles/mg. hr.) and the indicated quantity of mdCMP as substrate. dTTP (●) and dCTP (▲) were $10^{-4}$ M.

is inhibitory, while dCTP appears to activate the enzyme$^{11,13}$ (Fig. 1). Although the Lineweaver Burk plot in the presence of dTTP is suggestive of competitive inhibition, the lines intersect before the ordinate, and a $K_i$ cannot therefore not be determined. The $K_M$ for mdCMP, $3.3 \times 10^{-4}$ M, is of the same order of magnitude as the $K_M$ in other tissues.$^5$ In the presence of dCTP a lowering of the Michaelis constant from 3.3 to $1.8 \times 10^{-4}$ M was observed. A 28 to 43 per cent decrease in $K_M$ was found in two other experiments. dTTP does not inhibit dCMP deaminase at a pH below 7.4 (Fig. 2) but, as has been reported in other tissues, becomes inhibitory in the higher pH range.$^{14}$ Similarly, dCTP is more effective as an activator in the more alkaline pH range. Preincubation of the enzyme with dCTP prevented the inhibition by dTTP. These data do not completely rule out the possibility that the effect observed with dCTP may result from protection of the enzyme rather than its activation.

The effects of mercaptoethanol and pHMB on dCMP deaminase are shown in Table 3.$^{2,5}$ pHMB ($10^{-4}$ M) will result in 58 per cent inhibition of dCMP deaminase; this inhibition is prevented by an equimolar concentration of dCTP. A $2 \times 10^{-3}$ M concentration of 2-mercaptoethanol prevents the inhibition of dTTP of dCMP deaminase. As was first reported for the chick embryo enzyme,$^{15}$ Mg$^{++}$ ion ($10^{-3}$ M) decreases the dTTP inhibition of
leukocyte dCMP deaminase by 60 per cent. Mg\(^{++}\) does not potentiate activation by dCTP. dUTP, dCDP, dTDP and dUTP had no effect on dCMP deaminase.

No significant differences were detected in the dTTP inhibition of dCMP deaminase obtained from normal and leukemic cells. As illustrated in a typical experiment (Table 4), addition of dTTP resulted in similar degrees of inhibition of dCMP deaminase irrespective of the enzyme source. Higher concentrations of dTTP were needed for inhibition of dCMP deaminase in crude homogenates than for inhibition of the partially purified enzyme used in Table 3. We have previously reported that the level of dCMP deaminase is the same in normal and leukemic leukocytes.\(^7\)

*Properties of Nucleoside Deaminase*

As reported above, nucleoside deaminase is relatively stable to heat and to low pH. The crude enzyme is stable at -10 C. for at least 3 months. The pH activity curve of nucleoside deaminase with dC as the substrate is shown in Figure 3 and compared to that of dCMP deaminase. The former enzyme has a broader pH optimum, showing little decrease in activity at high pH. In crude homogenates, pHB in a concentration of 10\(^{-4}\) M resulted in 70 per cent in-
**Table 3.** Effect of dCTP, TTP and Sulfhydryl Compounds on dCMP Deaminase

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative Rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>dTTP (10⁻⁴ M)</td>
<td>5</td>
</tr>
<tr>
<td>dTTP followed by dCTP (10⁻⁴ M)</td>
<td>10</td>
</tr>
<tr>
<td>dCTP followed by dTTP</td>
<td>95</td>
</tr>
<tr>
<td>Mercaptoethanol (2 x 10⁻² M) followed by dTTP</td>
<td>58</td>
</tr>
<tr>
<td>pHMB (10⁻⁴ M)</td>
<td>14</td>
</tr>
<tr>
<td>dCTP (5 x 10⁻³ M) followed by pHMB</td>
<td>90</td>
</tr>
</tbody>
</table>

*The rate with dCMP as substrate with no additions to the mixture was set arbitrarily at 100. Enzyme from CLL cells purified to step 4 of Table 1 was used in this experiment. The enzyme protein in 0.05 M potassium phosphate buffer, pH 7.8, was preincubated at 23 C, for 10 minutes with either dTTP, dCTP, pHMB or mercaptoethanol. At the end of this period the other compounds were added in some tubes and the incubation continued for another 10 minutes. The reaction was started by the addition of substrate.

**Discussion**

Numerous examples of feedback control mechanisms by an end product of a biosynthetic pathway have been documented in bacteria. Relatively few such examples have been described in animal cells and none has been reported in human leukocytes. The enzymatic interconversions of the pyrimidine deoxyribonucleotides, shown in Figure 4, have been previously established in human leukocytes. It has been suggested that since deoxycytidylate is an efficient precursor of thymine, dCMP deaminase may play a role in the biosynthesis of DNA. Studies on dCMP deaminase from other tissues suggest that dTTP, the end product of this pathway, may control its own rate of synthesis through feedback inhibition of dCMP deaminase, the first enzyme in this biosynthetic sequence. This inhibition may be an allosteric effect where dTTP and dCTP, two end products of the metabolic pathway, react on a regulatory ("allosteric") site of the enzyme, which is distinct from the active center.
Table 4.—Inhibitory Effect of dTTP on dCMP Deaminase from Normal and Leukemic Leukocytes*

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Per Cent 10⁻³ M dTTP</th>
<th>Inhibition 2 × 10⁻⁴ M dTTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal 1</td>
<td>78</td>
<td>9</td>
</tr>
<tr>
<td>Normal 2</td>
<td>70</td>
<td>28</td>
</tr>
<tr>
<td>AL</td>
<td>88</td>
<td>23</td>
</tr>
<tr>
<td>CLL</td>
<td>68</td>
<td>24</td>
</tr>
</tbody>
</table>

* dTTP was added to the reaction mixture 10 minutes before dCMP. The supernatant of crude leukocyte homogenates was used for these experiments. The leukocyte preparations isolated from the patient with acute leukemia contained 95 per cent blasts, 2 per cent polymorphonuclear granulocytes and 3 per cent lymphocytes. The CLL preparation contained 99 per cent lymphocytes. The normal preparations consisted of 88 per cent polymorphonuclear granulocytes, 2 per cent eosinophils, 4 per cent monocytes and 6 per cent lymphocytes (Subject 1) and 84 per cent polymorphonuclear granulocytes, 6 per cent eosinophils and 10 per cent lymphocytes (Subject 2).

Fig. 3.—Activity of nucleoside deaminase as a function of pH. The buffer solutions were 0.1 M phosphate below pH 7.5, 0.1 M Tris HCl from pH 7.5 to pH 9, and 0.2 M carbonate above pH 9.

The action of dCTP, dTTP, Mg⁺⁺ and sulfhydryl agents is consistent with the hypothesis that these compounds act at the same site. The "allosteric" activator (dCTP) and inhibitor (dTTP) may effect the reaction rate through structural changes in enzyme configuration. This theory is supported by the observation that the sedimentation velocity of dCMP deaminase purified from the chick embryo is greater in the presence of dCTP than in the presence of dTTP.¹³
Table 5.—Activity of Nucleoside Deaminase in Normal and Leukemic Leukocytes

<table>
<thead>
<tr>
<th>Type of Cell</th>
<th>Number</th>
<th>dCMP Deaminase* Mean and Range</th>
<th>Nucleoside Deaminase* Mean and Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (granulocyte rich)</td>
<td>6</td>
<td>1.6 (1.0–2.4)</td>
<td>5.5 (3.5–10)</td>
</tr>
<tr>
<td>CML</td>
<td>3</td>
<td>1.2 (1.1–1.6)</td>
<td>2.8 (2.0–4)</td>
</tr>
<tr>
<td>CLL</td>
<td>5</td>
<td>1.0 (0.6–1.4)</td>
<td>0.2 (0–1.0)</td>
</tr>
<tr>
<td>Lymphocytes (thoracic duct)</td>
<td>2</td>
<td>1.3</td>
<td>0.3</td>
</tr>
<tr>
<td>AL</td>
<td>4</td>
<td>1.5 (0.6–2.5)</td>
<td>0.4 (0.1–0.7)</td>
</tr>
</tbody>
</table>

*Specific activity expressed as μmoles/hr./mg. protein.

Fig. 4.—Pyrimidine deoxyribonucleotide interconversions in human leukocytes.

Although the exact nature of this control mechanism remains uncertain, interaction of these nucleotides does occur in the human leukocyte. As has been described for other allosteric effects, deviation from classic kinetics was observed when the inhibitory effect of dTTP was investigated. This may explain the lack of linearity of the substrate-velocity curve in the presence of dTTP. In the presence of dCTP, a modest but reproducible lowering of the Michaelis constant is noted, suggesting that the allosteric activator functions by increasing the affinity of the enzyme for the substrate.

A comparison of the maximum velocities of dCMP deaminase and thymidylate synthetase in vitro reveals that the deamination of dCMP occurs at a rate over 1000 times greater than the formation of dTMP from dUMP. Although extrapolations from an in vitro to an in vivo situation must remain conjectural, being limited by the availability of cofactors, pool sizes and compartmentation, this ratio would be in keeping with Friedkin’s suggestion that thymidylate synthetase may have a rate-limiting function in this pathway.
In the presence of excess dTTP, however, dCMP deaminase could become rate-limiting.

The distribution of the two enzymes in various cell types is quite different. Thymidylate synthetase is detectable in the leukocytes of patients with AL, CML and infectious mononucleosis, but not in leukocytes from normal subjects. On the other hand, the specific activity of dCMP deaminase is approximately the same in the leukocytes of normal subjects and in those from patients with leukemia. The occurrence of appreciable activity of this enzyme in the mature granulocyte, an end-cell devoid of mitotic activity, may speak against the major function of dCMP deaminase being primarily in cell replication. Since the enzymes from normal and leukemic cells are equally susceptible to inhibition by dTTP, it appears that similar feedback controls are operative in cells at various stages of maturation.

The nucleoside deaminase, in contrast to dCMP deaminase, deaminates either the riboside or deoxyriboside of cytosine. It is active over a wide range of pH. In examining the findings obtained from assays of the level of nucleoside deaminase activity two facts emerge: more enzyme was present in myeloid cells than in lymphocytes, and only trace activity was present in the white cell preparations consisting mainly of blasts obtained from acute leukemia patients. Numerous lysosomes are found in mature granulocytes, which are not found in the lymphocytes or in earlier stages of granulocyte development. Since the deamination reactions catalyzed by this enzyme may be part of a catabolic sequence, a lysosomal localization could explain the cell-type distribution observed for this enzyme.

**SUMMARY**

The feedback control of dCMP deaminase, the first enzyme in a biosynthetic sequence of pyrimidine deoxyribonucleotide interconversions, has been characterized in leukocytes isolated from the peripheral blood of normal subjects and from patients with leukemia. As reported in other systems, dTTP, an end product of the pathway, may function as an allosteric inhibitor of this enzyme, while dCTP, another end product, prevents the dTTP inhibition.

Nucleoside deaminase is present in human leukocytes. Some of its properties are described.

**SUMMARIO IN INTERLINGUA**

Le regulation per reaction retrorse de deaminase de deoxycytidina-5'-PO₄, le prime enzyma in un sequentia biosynthetica de interconversiones de deoxyribonucleotida pyrimidinica, esseva characterisate in leucocytos isolate ab le sanguine peripheric de subjectos normal e de patientes con leucemia. Como reportate pro altere systemas, deoxycytidina-5'-triphosphato, un producto terminal del circuito, functiona possibilemente como inhibitor allosteric de iste enzyma, durante que deoxycytidina-5'-triphosphato, un altre producto terminal, preveni possibilemente le inhibition de deoxycytidina-5'-triphosphato.

Deaminase de nucleosida es presente in leucocytos human. Certes de su proprietates es describite.
REFERENCES

Regulatory Mechanisms in the Human Leukocyte: I. The Feedback Control of Deoxycytidylate Deaminase

ROBERT SILBER